

Rice root morphological and physiological traits interaction with rhizosphere soil and its effect on methane emissions in paddy fields

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ABSTRACT

Rice (*Oryza sativa* L.) paddies contribute approximately 7–17% to total global methane (CH₄) emissions and are considered an important source of human-induced climate change. However, the interactive effects of rice roots and soil microbes on CH₄ emissions in paddy fields are not clearly understood. We conducted two field experiments over three years. Soil CH₄ fluxes and cumulative CH₄ emissions, rice root traits, and microbial communities and activities in soil were measured using three mid-season *japonica* rice cultivars (Wuyujing 3, Zhendao 88, and Huaidao 5) that have the same growth durations and similar aboveground traits before heading. The CH₄ emissions during the mid-growing period (from panicle initiation to heading) contributed 39.0–49.7% of the total emissions during the entire growing season and differed significantly among the rice cultivars. The root morphological and physiological traits (i.e. root dry weight, root length, root oxidation activity, and root radial oxygen loss) were negatively correlated with CH₄ fluxes. Compared to the zero-N control, application rates of N fertilizer at 54 and 108 kg ha⁻¹ increased root biomass of cultivar Zhendao 88 by 10.1% and 17.3%, respectively, leading to corresponding decreases in CH₄ emissions by 12.7% and 22.9%. The root exudates (malic acid, succinic acid, and citric acid) promoted the abundance and activity of methanotrophs, which was the primary factors underlying the low CH₄ emissions in the paddy fields. Our findings suggested that stronger root systems, higher oxygen delivered by roots available for methanotrophs and suitable root exudates interacted in the rhizosphere, established a favourable habitat for microbial populations, and reduced CH₄ emissions in paddy fields.

1. Introduction

Methane (CH₄) is a significant greenhouse gas with a global warming potential that is 34 times greater than that of the equivalent mass of carbon dioxide in the atmosphere (IPCC, 2013). It is responsible for approximately 20% of the global warming effects (Kirschke et al., 2013). Rice paddies cover about 155 million hectares worldwide and contribute approximately 7–17% to global CH₄ emissions (Smith et al., 2008; Bridgman et al., 2013; IPCC, 2013). To reduce global CH₄ emissions, it is imperative to understand the patterns and controls of soil CH₄ emissions in paddy fields.

Rice plants may exert significant impacts on soil CH₄ emissions in paddy fields (Smith et al., 2008; Pittelkow et al., 2014; Malyan et al., 2016). Some aboveground morphological traits of rice, such as plant

height, tiller number and leaf area, have been shown to be positively correlated with soil CH₄ emissions (Wang et al., 1997; Aulakh et al., 2002). Developing large panicles benefits rice production for high yield and produces low CH₄ emission because rice variety with a large panicle size may allocate increased photosynthate to developing panicles, and reduce photosynthate allocation to the roots and soils, leading to the decrease in the substrate for CH₄ production (Das and Baruah, 2008a; Jiang et al., 2016). Jiang et al. (2017) estimated that increasing rice aboveground biomass by 10% could reduce annual CH₄ emissions from Chinese paddy fields by 7.1% based on a meta-analysis of the data from China's second state soil survey. There are reports that the aboveground traits have trivial effects on soil CH₄ emissions despite their potential effects on CH₄ transport from soil, and root traits may be the vital factors regulating soil CH₄ emission (Satpathy et al., 1998;

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Wassmann and Aulakh, 2000; Gutierrez et al., 2013; Zhang et al., 2015; Jiang et al., 2017).

Rice roots provide a direct link between aboveground plants and soil microbes which control soil CH₄ production. Increasing evidence has shown the pivotal role of rice roots in CH₄ emissions from paddy fields. For example, Wang et al. (1997) reported that the root air space and oxidation power adjust the CH₄ source strength and affect CH₄ emissions. A high oxygen-releasing capacity of plants can create an oxidized root area in the rhizosphere (Gutierrez et al., 2014). Jiang et al. (2017) believed that a high-yielding cultivar significantly increased root porosity and the abundance of methane-consuming microorganisms, suggesting that the larger and more porous root systems of high-yielding cultivars facilitated CH₄ oxidation by promoting O₂ transport to soils. Greater root growth provides a greater surface area for the diffusion of CH₄ into roots (Das and Baruah, 2008b). Root biomass, exudates and decaying root tissues greatly contribute to variations in CH₄ emissions from paddy soils (Setyanto et al., 2000; Malyan et al., 2016). Eighty to ninety percent of the total CH₄ emissions from rice fields occur via the root system (Watanabe et al., 1994; Das and Baruah, 2008b). However, the effects of rice root morphophysiological traits and exudates on CH₄ emissions and mechanisms are not clearly understood.

Rice cultivars exhibit great differences in root traits. Rice roots function as organs for water and nutrient uptakes and anchorage in soil. Roots also release a wide range of organic and inorganic compounds into the rhizosphere (Neumann and Römhild, 2007). Since CH₄ emissions are mainly determined by the net balance between the activities of methanogens and methanotrophs, any factors influencing these microbes could influence CH₄ metabolism (Conrad, 1996; Shrestha et al., 2010; Kai et al., 2011; Bridgman et al., 2013; Cai et al., 2016; Reim et al., 2017). Although both the diversity and activity of microbes in the rhizosphere related to CH₄ emissions have been extensively studied in numerous articles, the methods by which rice roots coordinate the dynamic interactions between soil microorganisms and plants in the rhizosphere and regulate CH₄ emissions in paddy fields have not been clarified.

In this study, we examined the dynamics of CH₄ emissions in paddy field ecosystems, rice root morphological and physiological traits, microbial diversity and density, and their influences on the CH₄ cycle in the paddy fields of three typical mid-season *japonica* rice cultivars. The major objectives were: 1) to investigate the interactive effects of rice roots and soil microbes on CH₄ emissions, and 2) to explore the impacts of nitrogen application on rice roots and CH₄ emissions in paddy ecosystems.

2. Materials and methods

2.1. Experimental site and design

2.1.1. Experiment 1

The field experiment was conducted on a research farm belonging to the Key Laboratory of Crop Genetics and Physiology of Yangzhou University, Jiangsu Province, China (32°30'N, 119°25'E) during the rice growing season (May to October) in 2014 and repeated in 2015. Three mid-season *japonica* rice cultivars, Wuyujing 3, Zhendao 88 and Huaidao 5, were selected from 15 *japonica* rice cultivars based on a field experiment from 2010 to 2013 in the city of Yangzhou. The entire growing duration and main aboveground agronomic traits of these three cultivars were nearly equivalent (Fig. S1). All cultivars headed (50% of plants) on 20–21 August and were harvested on 10–11 October. Over the two study years, seedlings were raised in a seedbed with a sowing date of May 15 and a transplant date of June 10 at a hill spacing of 0.16 m × 0.25 m, with two seedlings per hill. The experiment was laid out in a complete randomized block design with three replicates. The plot dimensions were 5 m × 4 m. The total nitrogen (N) application rate was 270 kg ha⁻¹, and it was applied as urea at the pre-

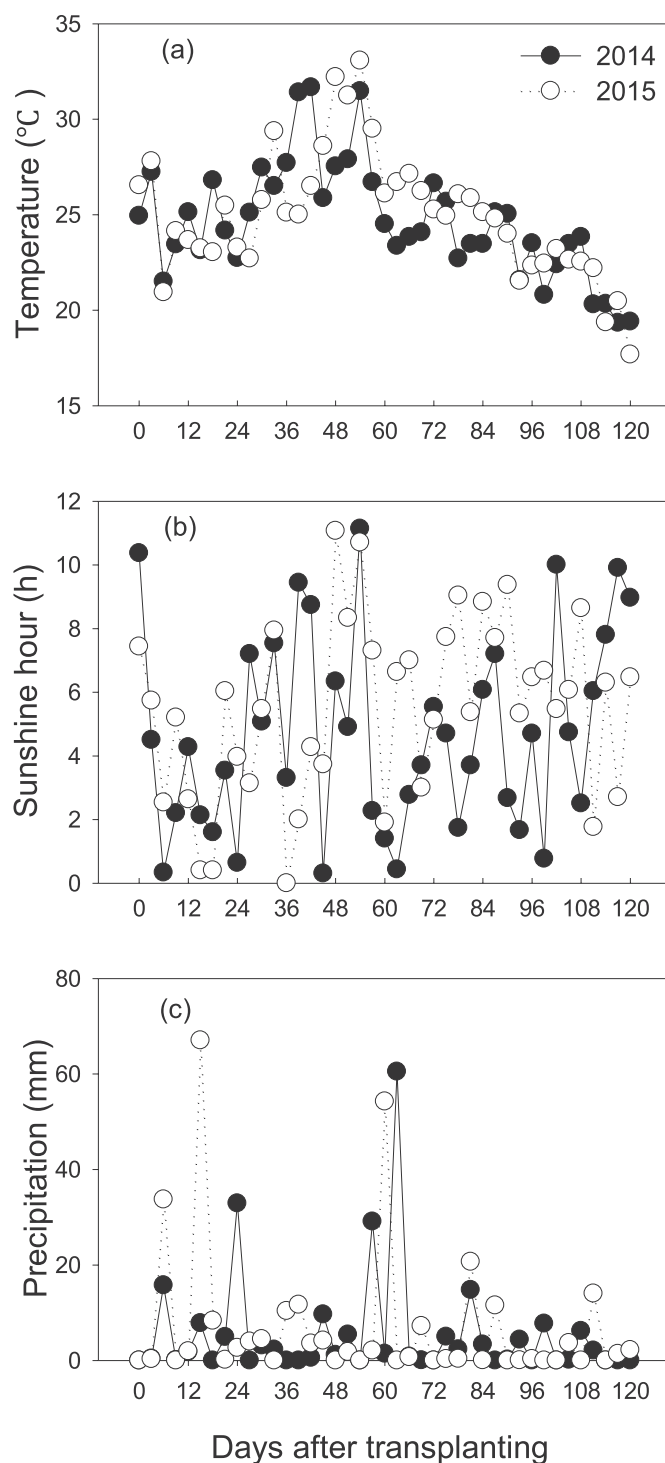


Fig. 1. Mean temperature (a), sunshine hours (b), and precipitation (c) during the growing seasons of rice in 2014 and 2015 at the experiment site of Yangzhou, Southeast China. Data are the means of every 3 days from the transplanting of rice.

transplanting (1 day before transplanting), early tillering (7 days after transplanting, DAT) and panicle initiation stages (38 DAT) in proportions of 50%, 10% and 40%, respectively. Phosphorus (40 kg ha⁻¹ as single superphosphate) and potassium (40 kg ha⁻¹ as KCl) were applied as basal fertilizer the day before transplanting.

The preceding crop was wheat (*Triticum aestivum* L.) in both years. The soil was a sandy loam [Typic fluvaquents, Eutisols (U.S. taxonomy)] with 23.2–25.1 g kg⁻¹ organic matter, 103–105 mg kg⁻¹ alkali

hydrolysable N, 33.7–35.8 mg kg⁻¹ Olsen-P, and 66.3–68.1 mg kg⁻¹ exchangeable K. The average air temperature, precipitation, and sunshine hours during the rice growing season across the two study years were measured at a weather station close to the experimental site (Fig. 1). The field was continuously flooded with 2–3 cm water until one week before harvest. Weeds, insects, and diseases controls were applied as needed.

2.1.2. Experiment 2

In 2016, the rice cultivar Zhendao 88 was used to test the effect of N application rates at the panicle initiation stage on root growth and CH₄ emission during the BT. No N fertilizer was applied at the pre-transplanting and the early tillering stages. Three N rates, i.e. 108 kg N ha⁻¹ (40% of 270 kg N ha⁻¹ applied at panicle initiation stage in experiment 1), 54 kg N ha⁻¹ and 0 kg N ha⁻¹ were only applied at the panicle initiation stage (38 DAT) in a complete randomized block design with three replicates. The plots were separated by ridges covered with plastic film. The other experimental details, such as the soil properties, experimental sites, seedling cultivation, hill spacing, plot size and management, were the same as those in experiment 1.

2.2. Methane emission measurements

CH₄ gas samples were collected using the static chamber method following Chu et al. (2015). The interval between the measurements was 3–5 days throughout the entire growing season. We installed three acrylic chambers in each plot for replication. For each flux measurement, gas samples were collected from 09:00 to 11:00 using a 20 ml syringe at 0, 10, 20, and 30 min after chamber closure. The concentrations of CH₄ from the collected gas samples were analysed by a gas chromatograph system (Agilent 7890A, Agilent Technologies, Palo Alto, CA, USA) and detected by a hydrogen flame ionization detector (FID). The carrier gas was argon-CH₄ (5%) at a flow rate of 40 ml min⁻¹. The temperatures for the column and ECD detector were maintained at 40 °C and 300 °C, respectively. The oven and FID were operated at 50 °C and 300 °C, respectively. Sample sets were rejected unless they yielded a linear regression value of r^2 greater than 0.90. The average fluxes and standard deviations (SDs) of CH₄ were calculated from four replicates. Growth stage cumulative and total CH₄ emissions during the growing season were determined using an integral function at each growth stage and over the entire growing season.

2.3. Plant sampling and measurements

At 5 days before booting period (–5 dBT, 50 DAT), BT (55 DAT) and 5 days after BT (5 dBT, 60 DAT), eight hills of plants were sampled for the measurement of root traits. For each root sampling, a block of soil (20 cm × 20 cm × 20 cm) around each individual hill was dug up using a soil sampler. The roots were carefully rinsed with a hydro-pneumatic device and detached from their nodal bases and then weighed to determine the fresh weight. Half of each root sample was used for the root length and root diameter measurements. The remaining roots were dried in an oven at 70 °C to a constant weight and weighed. To measure the root length and diameter, the roots were arranged and floated on shallow water in a glass tray (30 cm × 30 cm), scanned using a scanner (Epson Expression 1680 Scanner, Seiko Epson Corp., Tokyo, Japan) and analysed using a WinRHIZO Root Analysis System (Regent Instruments Inc., Quebec, Canada). Root pieces of approximately 5 mm were cut from 2 cm behind the root tip using a razor blade. The root tissues were vacuum infiltrated and fixed with 2.5% glutaraldehyde in 0.1 mol L⁻¹ phosphate buffer (pH 7.0) for 24 h. After washing with phosphate buffer, the sections were dehydrated in a graduated ethanol series and dried using a critical point dryer (CPD300, Leica EM). The dried root sections were glued onto specimen stubs, and the sections were coated with gold using a sputter coater. Finally, the root samples were viewed and photographed with a scanning electron

microscope (XL-30ESEM, Philips) (Yang et al., 2012). Three hills of rice roots were sampled to determine the root oxidation activity (ROA) by measuring the oxidation of alpha naphthylamine (α-NA) according to previously described methods (Liu et al., 2015), and the ROA was expressed as μg α-NA per gram DW per hour (μg α-NA g⁻¹ DW h⁻¹). Another three hills of rice roots were sampled to determine the root radial oxygen loss (ROL) using a citric acid colourimetric method (Kludze et al., 1993). The net rate of ROL in μmol O₂ plant⁻¹ d⁻¹ was calculated with the following formula: ROL = c (y - z), where, c = initial volume of Ti³⁺-citrate added to each test tube in L; and y = concentration of Ti³⁺-citrate solution of control (without plants) in μmol Ti³⁺ L⁻¹.

2.4. Soil sampling

Fresh soil was randomly collected from the centre of the rice root systems in the plots where the CH₄ flux measurements were performed, using a 2 cm diameter soil sampler, and roots and crop residues were removed. Each sample was mixed at least with nine soil cores to ensure that its weight was above 40 g. The soil cores were divided into two layers, the 0–2 cm depth (referred to as the surface soil) and 2–10 cm depth (referred to as the rhizosphere soil). We also included the unplanted plots in experiment 1, and the 2–10 cm depth in the unplanted plots was named “unplanted soil”. The samples were tetrasected: the first section was used for the microbial analysis (including qPCR and amplicon sequencing analysis); the second section was used to measure the methane production activity (MPA) and methane oxidation activity (MOA); the third section was used to analyse the soil pH; and the remaining section was stored at –80 °C.

2.5. Rhizosphere exudate collection, processing and analysis

The rhizosphere exudates of the rice plants were collected by a filter paper method (Theint et al., 2015). The plants were collected from the paddy fields together with the intact soil. After the soil around the roots was gently removed without injuring the roots, each individual plant was placed in a PVC pot (containing 800 ml distilled water) and incubated for 4 h at 30 °C. After collection, the exudate solution was filtered through a filter paper. The total organic carbon, carbohydrates and organic acids were determined as described by Aulakh et al. (2001).

2.6. DNA extraction from soils and quantitative PCR

The soil samples collected at the same time as the roots were lyophilized with a lyophilizer (LABCONCO, USA). DNA was extracted from the lyophilized soil samples using a FastDNA™ SPIN kit for soil (MP Biomedical, Santa Ana, CA, USA), and the tests were performed in triplicate. The quality and quantity of the DNA were assessed using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). A real-time quantitative PCR was run on an ABI 7500 real-time PCR system (Applied Biosystems) with three replicates for each sample. The PCR reaction contained 5 μl SYBR Premix (TaKaRa), 0.5 μl primer mix and 4.5 μl DNA template. The PCR amplification was performed with the following reaction conditions: initial denaturation at 95 °C for 30 s, 40 cycles of 95 °C for 10 s, annealing at 55 °C for 30 s and 72 °C for 30 s, followed by a final extension at 72 °C for 1 min. The primers were MLF (GGTATGGAGCAGTACGAGGAGTTC) and MLR (GTAGCCGAAGAAGCCGAGACG), A189F (GGTGACTGGGACTTCTGG) and mb661R (CCGGTGCAACGTCYTACC), MB10γ (GGTCCGAAGATC CCCCCTT) and 533r (GTGCCAGCAGCCGCGTAA), and MB9α (CCC TGAGTTATTCCGAAC) and 533r for the methanogens (*mcrA*), methanotrophs (*pmoA*), type I methanotrophs, and type II methanotrophs, respectively (Henckel et al., 1999; Lin et al., 2005; Luton et al., 2002; Juottonen et al., 2006).

2.7. Methane production activity and methane oxidation activity

The methane production activity (MPA) and methane oxidation activity (MOA) in the soil samples were determined as described by Krüger et al. (2001). For the MPA assay, 10 g fresh soil in triplicate was repeatedly flushed with N₂ and incubated for 48 h at 25 °C in the dark with shaking at 150 rpm. Five millilitre headspace gas samples were collected with pressure lock syringes to determine the CH₄ concentration. For the MOA assay, 5 g fresh soil in triplicate was placed into a 150 ml Erlenmeyer flask. The flasks were sealed with rubber stoppers, 1 ml air was pumped out of each flask and then 1 ml pure CH₄ was injected into the headspaces to generate an approximately 7500 ppm CH₄ concentration. The incubation was performed at 28 °C in the dark with shaking at 150 rpm for 24 h, and a flask without soils was used as the reference. Five millilitre headspace gas samples were collected to determine the CH₄ concentration. Two types of treatments were used: one was a root exudate treatment in which 10 ml of root exudates extracted from three rice cultivars was blended with 10 g unplanted soil samples; and the other treatment was an exogenous organic acids treatment, in which 10 ml of malic acid, citric acid, tartaric acid and succinic acid at concentrations of 0, 50, 100, 150 and 200 $\mu\text{mol L}^{-1}$ were blended with 10 g unplanted soil samples, respectively.

2.8. Amplicon sequencing and sequence processing

Soil DNA was extracted using a FastDNA™ SPIN kit for soil and diluted to 1 ng μL^{-1} . Each DNA extract was amplified to identify methanogens and methanotrophs with the same primers used in the qPCR. To distinguish amplicons originating from different samples, barcode oligonucleotides were ligated to the forward primers. The DNA libraries were constructed using a TruSeq DNA PCR-Free sample preparation kit, quantified by Qubit and qPCR, and then sequenced on a HiSeq 2500

(Illumina, USA) platform at Novogene (Beijing, China).

2.9. Statistical analysis

An analysis of variance (ANOVA) was performed using the SAS/STAT statistical analysis package (version 6.12, SAS Institute, Cary, NC). Data were tested with a post-hoc least significant difference (LSD) test at the $P < 0.05$ and $P < 0.01$ levels (LSD_{0.05} and LSD_{0.01}, respectively). Graphs were drawn using SigmaPlot 11.0 (SPSS Inc., Point Richmond, CA, USA). A correlation analysis was used to evaluate the relationships between the root and microbe traits and CH₄ emissions.

3. Results

3.1. Methane emission

In experiment 1, the seasonal patterns of CH₄ emissions were similar in all paddy fields and generally showed two peaks during the rice growing season in both 2014 and 2015 (Fig. 2). The CH₄ fluxes were low when rice was newly transplanted, increased sharply 5 days after transplanting (DAT) and reached the first peak at 14 DAT, which was followed by a dramatic decrease to 38 DAT and then a sharp increase. The fluxes reached the second peak from 50 to 60 DAT, which corresponded to the BT for three rice cultivars. Thereafter, the CH₄ fluxes declined gradually and were maintained at a relatively low level from 71 DAT (heading stage) to harvest (Fig. 2a and b).

The total cumulative CH₄ emissions during the entire growing season for the three rice cultivars were observed in the decreasing order of Wuyujing 3 > Zhendao 88 > Huaidao 5 (Fig. 2c and d), and significant differences were not observed among the cultivars during the early (0–38 DAT) and late (72 DAT to maturity) growing seasons. Although the mid-growing season (from panicle initiation to heading, 39

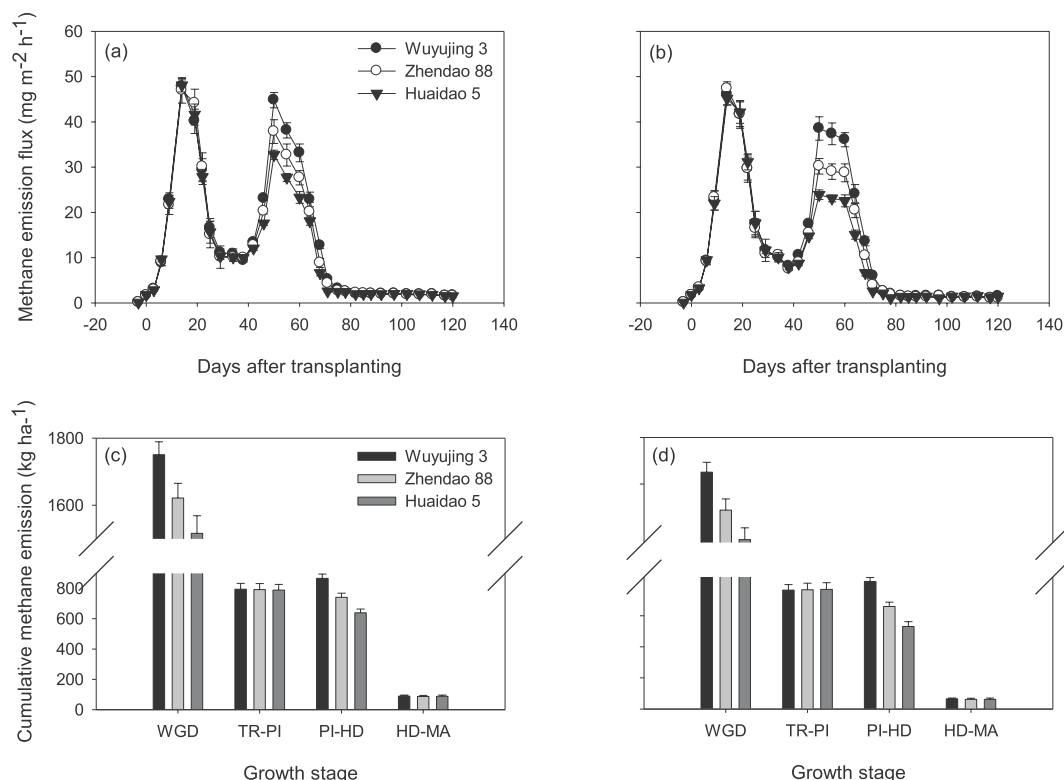


Fig. 2. Profiles of CH₄ emissions in the paddy fields of the three rice cultivars throughout the entire growing seasons in 2014 and 2015. (a) and (b) CH₄ emission fluxes in 2014 (a) and 2015 (b). (c) and (d) Cumulative CH₄ emissions in different stages and total emissions in 2014 (c) and 2015 (d). WGD: whole growth duration; TR-PI: from transplanting to panicle initiation (0–38 DAT); PI-HD: from panicle initiation to heading (39–71 DAT); HD-MA: from heading to maturity (72 DAT to maturity); DAT: Days after transplanting. The values are the means with standard errors (n = 3).

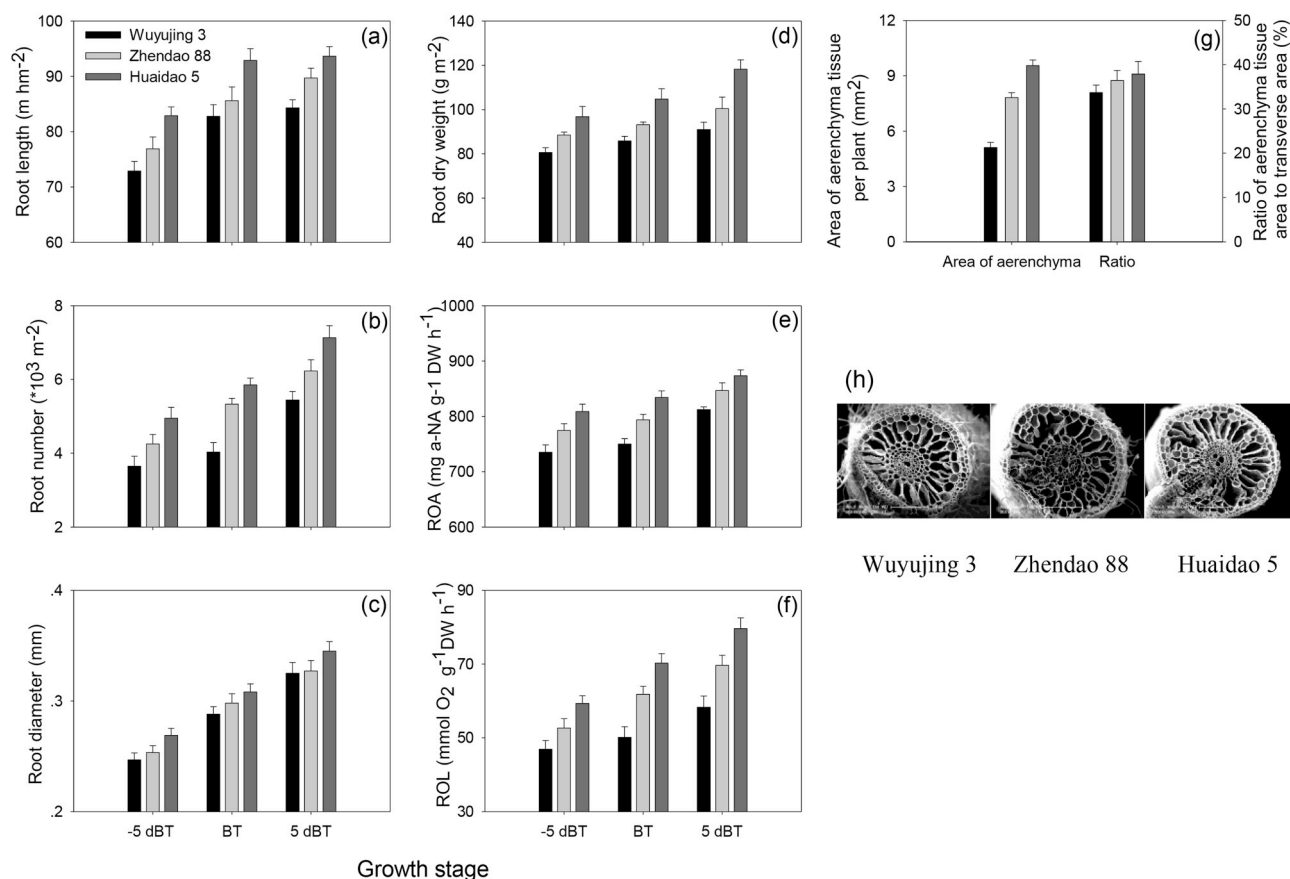


Fig. 3. Root morphological and physiological traits of the three rice cultivars during BT in 2015. (a) Root length. (b) Root number. (c) Root diameter. (d) Root dry weight. (e) Root oxidation activity (ROA). (f) Root radial oxygen loss (ROL). (g) Area of aerenchyma tissue and ratio of aerenchyma tissue area to transverse area. (h) Scanning electron micrographs of transverse sections from rice roots at 5 dBT. DW: Dry weight. Ratio: Ratio of aerenchyma tissue area to transverse area (%). Bar = 100 μm . The values are the means with standard errors ($n = 3$).

DAT to 71 DAT) only accounted for approximately 27% of the whole field growth duration, the CH_4 emissions from the paddy fields during this period were 49.5–49.7%, 44.3–45.7% and 39.0–42.1% of the total CH_4 emissions for the Wuyujing 3, Zhendao 88 and Huaidao 5 cultivars, respectively. Differences in cumulative CH_4 emissions in this mid-growing season greatly contributed to the differences of those among rice cultivars. The CH_4 fluxes during this period differed significantly among the cultivars, especially during the BT (Fig. 2a and b). Therefore, we conducted the root and soil trait assays three times around the BT at –5 dBT, BT and 5 dBT in 2015 to extrapolate their relationships with CH_4 emissions.

3.2. Morphological and physiological traits of rice roots

The morphological traits of the three rice cultivars, such as the root length, number, dry weight, and diameter, all increased from –5 dBT to 5 dBT. The physiological traits, including the ROA and ROL, showed the same trends. All the above traits at the same stages exhibited similar patterns, with Wuyujing 3 < Zhendao 88 < Huaidao 5 (Fig. 3a, b, c, d). The area of aerenchyma tissue per plant and the ratio of aerenchyma tissue area to transverse area at 5 dBT were the highest in Huaidao 5 (Fig. 3g and h). Methane emissions were significantly and negatively correlated with the root length, number and dry weight, with r values ranging from -0.845^{**} to -0.698^* (Figs. S2a, b, d). The root diameter of Huaidao 5 was slightly larger than that of Wuyujing 3 during the BT period (Fig. 3c), and a statistical correlation was not observed between the root diameter and CH_4 fluxes (Fig. S2c). The ROA and ROL also showed significantly negative relationships with the CH_4 fluxes (r values of -0.779^{**} and -0.800^{**} , respectively, Figs. S2e and f).

3.3. Root exudates

An analysis of the organic carbon excreted from roots showed that during the BT period, the mean total organic carbon (TOC) exudation rates ranged from 27.87 to 34.85 mg C per plant. Huaidao 5 showed the highest release of TOC (Table 1). The low molecular weight organic acid species in the root exudates, including malic acid (MA), tartaric acid (TA), succinic acid (SA), citric acid (CA), acetic acid (AA) and oxalic acid (OA) were measured. Significant differences in the total organic acid (TOA) amount were not observed among the rice cultivars, although variations in the quantity of individual organic acids in the TOA were observed (Table 1). MA was present in conspicuously large amounts ($60.55\text{--}86.75 \mu\text{mol g}^{-1} \text{DW}$). The levels of MA, SA and CA showed similar patterns in the order of Huaidao 5 > Zhendao 88 > Wuyujing 3. The amounts of TA, AA and OA showed no differences among the cultivars. The statistical analysis showed that the CH_4 fluxes exhibited significantly negative correlations with the MA, SA and CA contents ($r = -0.845^{**} \sim -0.778^{**}$), and they did not correspond to the TOC, TOA and carbohydrate (CAR) content during the BT (Fig. S3).

In experiment 2, N fertilizer application at panicle initiation (38 DAT) had significant effects on the rice root traits and CH_4 fluxes at the BT (55 DAT) in Zhendao 88 (Table 2). The root dry weight, ROA and MA, CA and SA contents exuded from the roots were increased and the CH_4 fluxes were decreased by the N application. Compared to the zero-N control, application rates of N fertilizer at 54 and 108 kg ha^{-1} increased root biomass of cultivar Zhendao 88 by 10.1% and 17.3% and decreased CH_4 emissions by 12.7% and 22.9%, respectively. The results from this experiment further confirmed the negative correlation

Table 1

Contents of the root exudate components in three rice cultivars in 2015.

Stage	Cultivar	TOC	CAR	TOA	MA	TA	SA	CA	AA	OA
– 5 dBT	Wuyujing 3	27.87a	18.63a	6.53a	60.55c	33.3a	13.69b	16.84c	1.22c	5.44a
	Zhendao 88	28.22a	20.16a	7.09a	69.63b	30.11b	13.74b	20.12b	2.07b	6.22a
	Huaidao 5	28.69a	20.25a	7.47a	76.33a	20.69c	20.13a	23.57a	2.93a	3.19b
BT	Wuyujing 3	29.19b	19.69a	7.64a	65.96c	42.89a	15.57b	19.43c	3.91a	7.23a
	Zhendao 88	28.43b	17.32a	8.18a	74.44b	39.69a	15.69b	23.92b	3.17a	6.07b
	Huaidao 5	34.37a	22.68a	8.60a	80.16a	31.26b	24.11a	26.75a	1.83c	5.31c
5 dBT	Wuyujing 3	30.03b	19.48b	8.04a	72.15c	40.2a	17.2b	22.73c	1.52a	6.08a
	Zhendao 88	34.85a	18.15b	8.80a	83.20b	39.4a	18.02b	26.22b	1.25a	5.66a
	Huaidao 5	34.30a	24.87a	9.16a	86.75a	29.5b	28.15a	28.91a	1.29a	5.03a

TOC: total organic carbon; CAR: carbohydrate; TOA: total organic acid; MA: Malic acid; TA: Tartaric acid; SA: Succinic acid; CA: Citric acid; AA: Acetic acid; and OA: Oxalic acid.

The units are mg C plant^{−1} for TOC, CAR and TOA and μmol g^{−1} DW for individual organic acids.

Different letters (a, b and c) in the same column represent significant differences at the $P < 0.05$ probability level among cultivars at the same stage.

Table 2

Effects of N application rates on the root traits, methane emission fluxes and organic acids exuded from rice roots at BT in Zhendao 88 in 2016.

N rate (kg ha ^{−1})	Root dry weight (g m ^{−2})	Root oxidation activity (mg α-NA g ^{−1} DW h ^{−1})	MA (μmol g ^{−1} DW)	CA (μmol g ^{−1} DW)	SA (μmol g ^{−1} DW)	CH ₄ flux (mg m ^{−2} h ^{−1})
0	72.3c	650.3c	60.2b	17.8b	11.2b	30.6a
54	79.6b	700.5b	65.9a	18.9b	12.9a	26.7b
108	84.8a	740.5a	68.2a	20.3a	13.7a	23.6c

Different letters (a, b and c) in the same column represent significant differences at the $P < 0.05$ probability level.

between the root traits and CH₄ emissions in experiment 1.

3.4. Methanogen and methanotroph communities and activity in paddy fields

The copies of the *16SrDNA* gene in bacteria and the *mcrA* gene in the methanogens from – 5 dBT to 5 dBT did not vary among the rice cultivars and growth stages (Fig. 4a and b). In contrast, the abundance of the *pmoA* gene in the methanotrophs increased from – 5 dBT to 5 dBT (Fig. 4c). Furthermore, the *pmoA/mcrA* ratios were significantly different among the three cultivars, with the highest abundance detected in the Huaidao 5 paddy soil (Fig. 4d). The number of *pmoA* copies and *pmoA/mcrA* ratios were negatively correlated with the CH₄ fluxes (Fig. S4).

Based on the amplicon sequencing, the methanogen and methanotroph communities were analysed on *mcrA* and *pmoA* genes from the rhizosphere soil samples of the Wuyujing 3, Zhendao 88 and Huaidao 5 cultivars. According to the observed OTU (operational taxonomic unit) richness, the Chao estimator and Shannon index indicated that the α-diversity did not differ in the methanogen communities but the methanotroph communities varied among the paddy soils (Table S1).

The *mcrA* and *pmoA* gene sequencing reads were classified at the genus level to investigate the changes in the methanogen and methanotroph communities at the BT. The methanogen classification showed that approximately 90% of the methanogen reads fell into the four genera *Methanobacterium*, *Methanosarcina*, *Methanoregula* and *Methanospaera* (Fig. 4e). The genera *Methylosarcina* and *Methylomonas* were detected as the dominant type I methanotrophs, which accounted for nearly 60% of the total methanotrophs, and the relative abundance of the two methanotrophs was the highest in the Huaidao 5 rhizosphere (Fig. 4f). The type II methanotrophs *Methylocystis* and *Methylosinus* accounted for less than 10% of the total methanotrophs and showed the lowest abundance in the Huaidao 5 rhizosphere. In the Huaidao 5 and Wuyujing 3 rhizospheres, 13.5% and 6.6% of the genera were unclassified, respectively.

Although the MPA was highest in the rhizosphere soil, but significant differences were not observed among the three cultivars in all soil types (Fig. 5a and b). Noticeably, after 24 h of incubation, significantly higher MOA was observed in the Huaidao 5 rhizosphere soil

compared with the Zhendao 88 and Wuyujing 3 rhizosphere soil and the surface soil (Fig. 5e and f). Then, we treated the soil without planted rice with three types of root exudates and exogenous organic acids at the BT. The MPA profiles exhibited a similar pattern to those in the untreated soils among the cultivars and exogenous acids (Fig. 5c and d). When the unplanted soil was blended with the root exudates, the MOA profiles in the headspace microcosms were consistent with the paddy field conditions and presented in the order of Huaidao 5 > Zhendao 88 > Wuyujing 3 (Fig. 5g), and they were intensified by low concentrations of MA, SA and CA and inhibited by high concentrations of these organic acids. The MOA was increased by 180% when treated with 100 μmol L^{−1} MA (Fig. 5h).

After the MOA assay, the microbial DNA was extracted from the soil samples and subjected to a quantitative PCR assay. The *pmoA* gene copies indicated that methanotroph growth was stimulated by root exudates and 100 μmol L^{−1} exogenous organic acids. The gene copies of methanotrophs were 2.8 times and 2.3 times of those of the control when treated with exudates from Huaidao 5 (Figs. 5i) and 100 μmol L^{−1} MA (Fig. 5j), respectively. The increased methanotroph growth greatly contributed to the type II methanotroph growth, and the gene copies of type II methanotroph were enhanced by 3.2 times and 2.6 times under the Huaidao 5 (Figs. 5i) and 100 μmol L^{−1} MA treatments (Fig. 5j), respectively. These results from the laboratory experiment confirmed the roles of root exudates, especially the individual organic acids, in regulating CH₄ emissions as presented in experiment 1.

4. Discussion

Methane is emitted from paddy fields via several biological processes including its production, oxidation and transportation through rice plants (Malyan et al., 2016). While the effect of rice plants on CH₄ emission has been widely studied, information on the interactions between rice roots and soil microbes on CH₄ emissions is still lacking. By measuring soil CH₄ emissions, root traits, and soil microbial composition and activity, we showed that 1) CH₄ emissions during the mid-growing period (from panicle initiation to heading) contributed 39.0–49.7% to the total emissions and were the main reason for the differences in CH₄ emissions among rice cultivars; 2) Root traits, rather than aboveground traits, regulated CH₄ emissions; and 3) Malic,

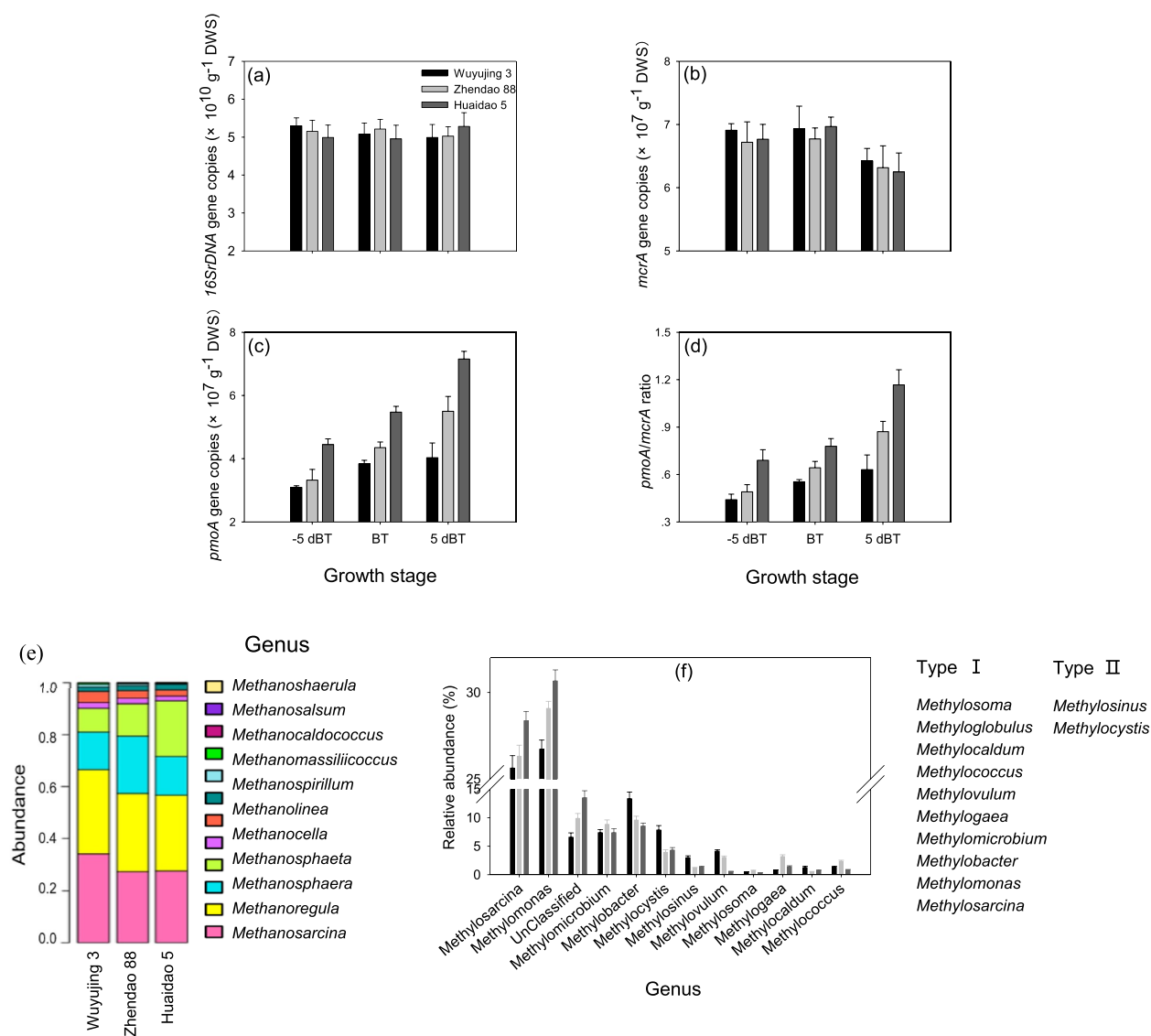


Fig. 4. Changes in the quantity of bacteria, methanogens and methanotrophs of the three cultivars during BT in 2015. (a) 16SrDNA gene copy numbers for bacteria. (b) *mcrA* gene copy numbers for methanogen quantification. (c) *pmoA* gene copy numbers for methanotroph quantification. (d) Ratio of *pmoA* to *mcrA*. (e) and (f) Genera distribution statistics of the methanogens and methanotrophs based on *mcrA* and *pmoA*, respectively. The sequencing reads of *pmoA* and *mcrA* genes were assigned to their taxonomic affiliations of methanogens (e) and methanotrophs (f), respectively, by the complete linkage clustering in the RDP functional gene pipeline and BLASTP comparisons in GenBank. DWS: Dry weight of soil.

succinic and citric acids in root exudates improved the abundance and activity of methanotrophs in rhizosphere soil, promoted CH_4 oxidation, and reduced CH_4 emissions in paddy fields.

4.1. Patterns of methane emissions during the growing season in three paddy fields

The profiles of CH_4 emissions in experiment 1 showed two peaks during the rice growing season in all three paddy fields (Fig. 2) (Das and Baruah, 2008a; Li et al., 2011; Chu et al., 2015). The first peak occurred at the mid-tillering stage (14 DAT) was mostly associated with fermentation during the decomposition of easily degradable soil organic matter and the suitable flooded conditions for methanogens after transplanting (Ma et al., 2009; Li et al., 2011). No significant differences in CH_4 emissions were observed among the rice cultivars because of the subtle differences in plant growth at this stage (Fig. 2) (Li et al., 2011). Furthermore, no obvious CH_4 emission peak was found before 15 DAT in the pot experiment (data not shown) as the rice seedlings were transplanted onto soil sieved with a 6 mm mesh and flooded for 10

days before transplantation. These results indicated that the first emission peak of CH_4 in field study (experiment 1) was mainly caused by crop residue fermentation with the shift of dry fields to flooded conditions.

The second CH_4 emission peak occurred from 39 to 71 DAT in experiment 1, which coincided with the panicle differentiation stage. This is a key developmental stage in rice while plant switches from vegetative to reproductive development, and allocates the photoassimilates to growing spikelets and kernels (Streck et al., 2009). Rice plants usually need more water and nutrients and are sensitive to the external environmental conditions (Quinones et al., 2017). This time period usually lasts 30–35 days for most rice cultivars, which accounts for approximately 1/4 of the whole field growth duration. However, the CH_4 emission during this period was 39.0–49.7% of the total cumulative emissions during the entire growing season. In addition, the mean CH_4 fluxes and cumulative emissions during this period differed significantly among the three cultivars (Fig. 2). During the late growing season (from the heading stage to maturity), the CH_4 emissions were much lower and no significant differences were observed among the

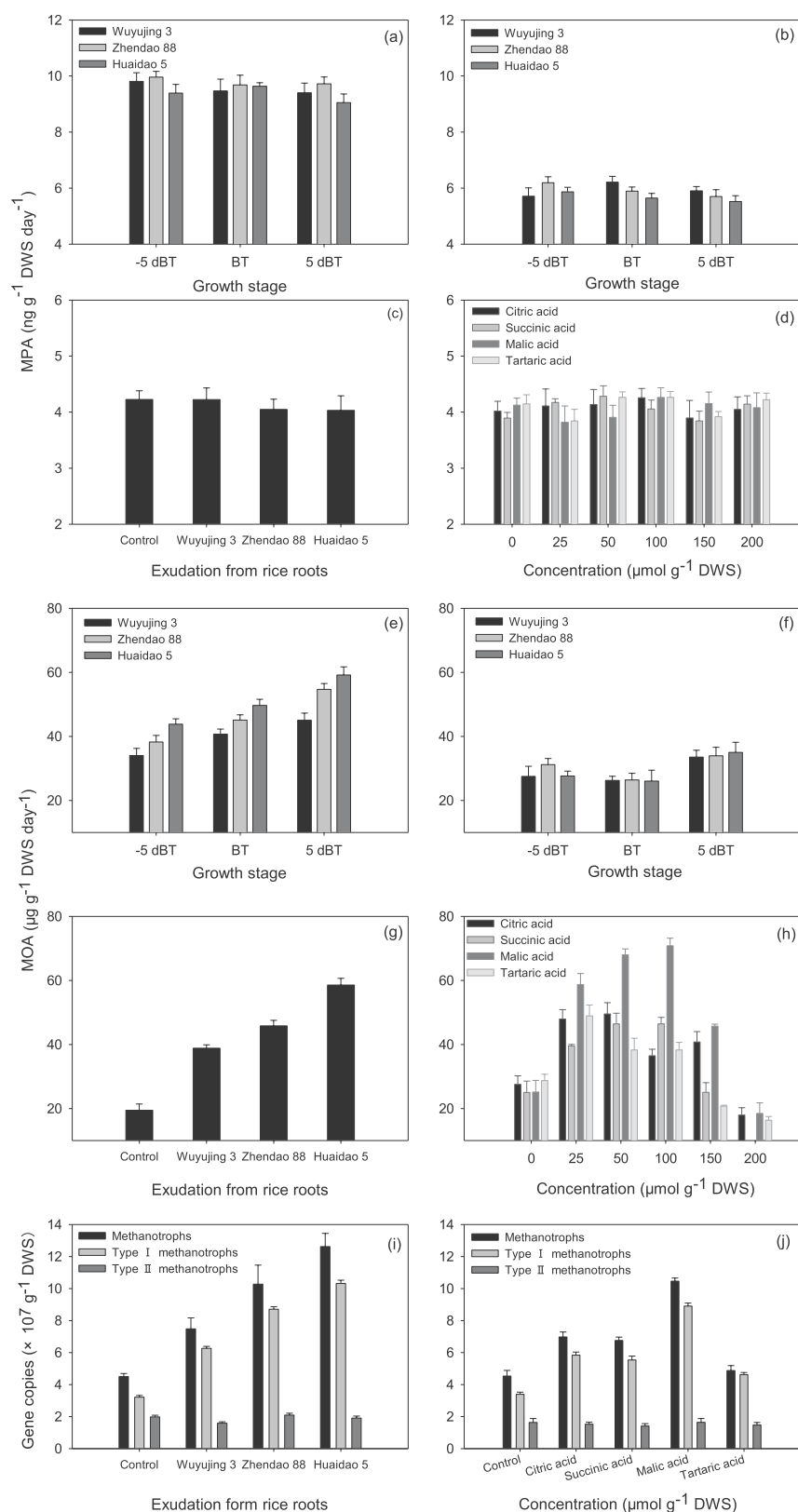


Fig. 5. Methane production activity (MPA) and methane oxidation activity (MOA) in 2015. (a) MPA of the rhizosphere soil from -5 dBT to 5 dBT. (b) MPA of the surface paddy soils from -5 dBT to 5 dBT. (c) MPA of the unplanted soils treated with root exudates from three rice cultivars at BT. (d) MPA of the unplanted soils treated with exogenous organic acids at BT. (e) MOA of the rhizosphere soil from -5 dBT to 5 dBT. (f) MOA of the surface paddy soils from -5 dBT to 5 dBT. (g) MOA of the unplanted soils treated with root exudates from three rice cultivars at BT. (h) MOA of the unplanted soils treated with exogenous organic acids at BT. (i) Changes in the *pmoA* gene copy numbers for methanotrophs in the unplanted soil samples treated with root exudates from the three cultivars. (j) Changes in the *pmoA* gene copy numbers for methanotrophs in the unplanted soil samples treated with 100 $\mu\text{mol L}^{-1}$ exogenous organic acids.

rice cultivars. These results implied that efforts should be focused on early and mid-growing season, particularly the mid-growing season in order to reduce the CH_4 emission in paddy fields.

4.2. Root morphological and physiological traits played more important roles in methane emissions in paddy fields than the aboveground plant traits

The main aboveground agronomic traits, such as the tiller number, plant height, leaf area index, and biomass were similar among the three rice cultivars during the BT (Fig. S1). However, there were significant

differences in the belowground traits, e.g., root dry weight, root length, ROA and ROL. These root traits were significantly and negatively correlated with CH₄ fluxes during the same stage (Fig. S2). These results showed that the root traits, rather than the aboveground traits, were responsible for the variations of CH₄ emissions among rice cultivars. We also found that the grain yield of Huaidao 5 was significantly higher than Zhendao 88 and Wuyujing 3, which was mainly attributed to its larger number of spikelets per panicle (Table S2; Zhang et al., 2009, 2013). Higher root weight and ROA during panicle differentiation promote the development of young panicles and the formation of large panicles (Liu et al., 2013; Zhang et al., 2013). Many researchers reported that root weight, ROA and LAI from heading to maturity were positively correlated with grain yield (Liu et al., 2015; Meng et al., 2016; Gu et al., 2017; Wang et al., 2018). Similar results were found in our present study (Fig. S5, Table S2). Stronger root systems from panicle initiation to heading not only improved rhizosphere environment for methanotroph growth and reduced CH₄ emissions, but also contributed to plant aboveground growth after heading and yield increase.

We further explored the potential reasons of low CH₄ emissions in the Huaidao 5 paddy fields, especially during the mid-growing season. A stronger root system could transport more oxygen into the soil, maximize soil aeration, and maintain high root oxidation, thereby increasing the rhizosphere soil Eh (Oxidation-reduction potential) and contributing to more suitable conditions for methanotroph growth and CH₄ oxidation (Fig. 5, S6, and Table S3). Higher ROA and ROL in Huaidao 5 increased the root biomass, delayed root senescence, and decreased the amount of aging and withering organizations of rice plants, leading to the decrease in the main substrates for methanogenic bacteria and finally a reduction of CH₄ production (Mitra et al., 2005; Zhang et al., 2009). Therefore, this concurrent changes of decreased CH₄ production and increased CH₄ oxidation promoted by the strong root system and enhanced oxygen availability contributed at least partly to reduced CH₄ emissions in paddy fields.

The management of rice agriculture via fertilizer application impacts the rice yield and CH₄ fluxes (Kai et al., 2011). In China and other developing countries, to obtain a high panicle number to increase grain yield, farmers usually apply excessive N fertilizer in the early rice growing season. However, this practice can aggravate N loss and increase unproductive tillers in the mid-growing season, thereby leading to lower root biomass and activity. Optimizing N application was able to improve the rice root traits and enhance the grain yield (Liu et al., 2013) and had the potential to reduce CH₄ emissions in paddy fields (Table 2). Rice genetic improvements over the past 70 years in China gradually increased the grain yield, and root weight and activity (Liu et al., 2015). Thus, selecting modern cultivars with high root biomass and activity or optimizing fertilizer application will be useful for decreasing the CH₄ emissions during rice production and will provide beneficial effects on global climate change.

4.3. Organic acids excreted from roots promoted methane oxidation and reduced methane emissions

TOC is the major source for CH₄ production by methanogens, and it is mainly derived from rice root exudates and plant parts (Lu et al., 2000). TOC was not correlated with CH₄ emissions in experiment 1, indicating that TOC might equally affect methanogens and methanotrophs, which offset each other. In addition, different components of TOC may function individually in regulating rhizosphere microbial communities. MA and CA are suitable carbon sources for many microorganisms (López-Bucio et al., 2000), and methanotrophs also grow on multi-carbon compounds, such as acetate, ethanol, malate, succinate and pyruvate (Rahman et al., 2011), which could be supplied by root exudates. Our results confirmed that the stronger root system and higher oxygen delivered by roots for methanotrophs (Fig. 3) stimulated root aerobic respiration, accumulated CA, MA and SA contents (Table 1), and changed the soil pH and Eh (Fig. S6). Similar results were

reported by Theint et al. (2015) who found that the significantly higher concentrations of MA and TA in rhizosphere exudates favour CH₄ emissions. A mild acidic solution can improve the soil ion exchange capacity and release P, Ca and Mg into the soil (Silber et al., 2010), which can further significantly increase the population and activity of methanotrophs (Table S3; Neumann and Römhild, 2007). He et al. (2016) reported a close link between high CH₄ fluxes and decreases in the MA and TA contents and increases in the SA and OA contents in the root exudates. Therefore, we concluded that higher organic acid contents, especially MA, CA and SA, played a vital role in the interactions between the roots and rhizosphere soil and led to CH₄ mitigation in paddy fields.

The net CH₄ emissions from rice paddies are determined by the balance between the activity of methanogens and methanotrophs (Conrad, 2007). The CH₄ oxidation in paddy fields is localized near surface aerobic layer and in the rhizosphere (Wassmann and Aulakh, 2000). We found that although the numbers of *mcrA* gene copies and the communities of methanogens were similar (Fig. 4b, e), significant and negative relationships were observed between the CH₄ fluxes and *pmoA/mcrA* ratio (Fig. 4d, Fig. S4d). Furthermore, the Huaidao 5 roots promoted type I dominant methanotroph abundance, especially for the genera *Methylocystis* and *Methylosinus*, and inactivated type II methanotroph abundance, indicating that the Huaidao 5 root system could create a more suitable habitat for aerobic type I methanotrophs (Fig. 4f). In addition, a number of unclassified methanotrophs displayed conspicuous stimulation in Huaidao 5 but were inhibited in Wuyujing 3, which might also be responsible for the depletion of CH₄ emissions (Fig. 4f). The results confirmed that the organic acids excreted from rice roots, especially MA, CA, and SA represented the anticipated scenario capable of reducing CH₄ emissions during rice production. The relative amount of organic acids required to conduct specific functions remains to be calculated and should be further investigated.

5. Conclusion

This study showed that the CH₄ emissions during the mid-growing period contributed 39.0–49.7% to the total cumulative emissions during the entire growing season in paddy fields and differed significantly among rice cultivars. The rice root morphological and physiological traits rather than the aboveground traits actually regulated the CH₄ emissions. Larger root system, more oxygen delivered by roots available for methanotrophs, and suitable contents of malic acid, citric acid and succinic acid in root exudates created favourable habitats for methanotroph population, increased the density and activity of methanotrophs, and promoted CH₄ oxidation. Increasing N application rate at panicle initiation stage increased root activity and the contents of malic acid, citric acid and succinic acid in root exudates, and decreased CH₄ fluxes during mid-growing period. Stronger root systems from panicle initiation to heading not only improved rhizosphere environment for methanotroph growth and reduced CH₄ emissions, but also contributed to plant aboveground growth after heading and yield increase. Our results suggested that either screening cultivars with strong root systems or increasing N inputs in panicle initiation stage may be a win-win strategy, as it simultaneously increased grain yield and decreased CH₄ emissions in paddy ecosystems.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.soilbio.2018.11.015>.

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